The NO-donor MPC-1011 stimulates angiogenesis and arteriogenesis and improves hindlimb ischemia via a cGMP-dependent pathway involving VEGF and SDF-1

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Abstract: Background and aims: Peripheral arterial disease (PAD) is an important cause of morbidity and mortality with little effective medical treatment currently available. Nitric oxide (NO) is crucially involved in organ perfusion, tissue protection and angiogenesis. Methods: We hypothesized that a novel NO-donor, MPC-1011, might elicit vasodilation, angiogenesis and arteriogenesis and in turn improve limb perfusion, in a hindlimb ischemia model. Hindlimb ischemia was induced by femoral artery ligation in Sprague-Dawley rats, which were randomized to receive either placebo, MPC-1011, cilostazol or both, up to 28 days. Limb blood flow was assessed by laser Doppler imaging. Results: After femoral artery occlusion, limb perfusion in rats receiving MPC-1011 alone or in combination with cilostazol was increased throughout the treatment regimen. Capillary density and the number of arterioles was increased only with MPC-1011. MPC-1011 improved vascular remodeling by increasing luminal diameter in the ischemic limb. Moreover, MPC-1011 stimulated the release of proangiogenic cytokines, including VEGF, SDF1 and increased tissue cGMP levels, reduced platelet activation and aggregation, potentiated proliferation and migration of endothelial cells which was blunted in the presence of soluble guanylyl cyclase inhibitor LY83583. In MPC-1011-treated rats, Lin-/CD31+/CXCR4+ cells were increased by 92.0% and Lin-/VEGFR2+/CXCR4+ cells by 76.8% as compared to placebo. Conclusions: Here we show that the NO donor, MPC-1011, is a specific promoter of angiogenesis and arteriogenesis in a hindlimb ischemia model in an NO-cGMP-VEGF- dependent manner. This sets the basis to evaluate and confirm the efficacy of such therapy in a clinical setting in patients with PAD and impaired limb perfusion.

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The NO-donor MPC-1011 stimulates angiogenesis and arteriogenesis and improves hindlimb ischemia via a cGMP-dependent pathway involving VEGF and SDF-1α

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HIGHLIGHTS

- PAD is a debilitating disease caused by abnormal narrowing of arteries with little or no medical treatment options existing.
- Novel and unique NO donor, MPC-1011, promotes angiogenesis via NO-mediated signaling and proliferation of endothelial cells.
- Thus, MPC-1011 might be successfully used in clinical setting in patients with PAD or in those with impaired limb perfusion.

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ABSTRACT

Background and aims: Peripheral arterial disease (PAD) is an important cause of morbidity and mortality with little effective medical treatment currently available. Nitric oxide (NO) is crucially involved in organ perfusion, tissue protection and angiogenesis.

Methods: We hypothesized that a novel NO-donor, MPC-1011, might elicit vasodilation, angiogenesis and arteriogenesis and in turn improve limb perfusion, in a hindlimb ischemia model. Hindlimb ischemia was induced by femoral artery ligation in Sprague-Dawley rats, which were randomized to receive either placebo, MPC-1011, cilostazol or both, up to 28 days. Limb blood flow was assessed by laser Doppler imaging.

Results: After femoral artery occlusion, limb perfusion in rats receiving MPC-1011 alone or in combination with cilostazol was increased throughout the treatment regimen. Capillary density and the number of arterioles was increased only with MPC-1011. MPC-1011 improved vascular remodeling by increasing luminal diameter in the ischemic limb. Moreover, MPC-1011 stimulated the release of proangiogenic cytokines, including VEGF, SDF1α and increased tissue cGMP levels, reduced platelet activation and aggregation, potentiated proliferation and migration of endothelial cells which was blunted in the presence of soluble guanylyl cyclase inhibitor LY83583. In MPC-1011-treated rats, $Lin^-/CD31^+/CXCR4^+$ cells were increased by 92.0% and $Lin^-/VEGFR2^+/CXCR4^+$ cells by 76.8% as compared to placebo.

Conclusions: Here we show that the NO donor, MPC-1011, is a specific promoter of angiogenesis and arteriogenesis in a hindlimb ischemia model in an NO-cGMP-VEGF- dependent manner. This sets the basis to evaluate and confirm the efficacy of such therapy in a clinical setting in patients with PAD and impaired limb perfusion.

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1. Introduction

Although coronary artery disease and stroke are the leading causes of death worldwide [1,2], many people also suffer from peripheral arterial disease (PAD) characterized by impaired blood flow to the lower extremities, claudication pain and severe exercise intolerance [3]. The most common cause of peripheral artery disease is atherosclerosis, however, less common causes include inflammatory disorders of the arterial wall, vasculitis, and noninflammatory arteriopathies [4]. Of note, most studies using revascularization strategies [5–7] or pharmacological treatments [8,9] indicate that patients with PAD have a markedly increased risk for death as well as cardiovascular events including critical limb ischemia with potentially life-changing limb loss.

Occlusive atherosclerosis occurs as a last step of endothelial nitric oxide (NO) deficiency, hence limiting signal transduction necessary for normal vascular function [10]. NO is ubiquitous molecule able to diffuse across cell membranes, modulating many physiological responses including gene expression, apoptosis, platelet function, vascular smooth muscle cell relaxation and proliferation, neurotransmission, memory, and immune stimulation [11–15]. Thus, delivery of NO appears to be an attractive therapeutic option in several cardiovascular conditions, including PAD, and this delivery is achieved through NO-releasing drugs. Those are pharmacologically active compounds that release NO in *vitro* and *in vivo*, e.g. sodium nitroprusside [16], organic nitrates and nitrite esters [17] and S- nitrosothiols [18]. Such NO-releasing drug is MPC-1011 (mirandaPharmaceuticals^{R} Ltd., Basel, Switzerland), an organic nitrate with NO-releasing properties. Unlikely to other organic nitrates, MPC-1011 does not induce either *in vitro* tolerance, *in vivo* cross tolerance, *or* oxidative stress *in vitro* and *in vivo* [19,20]. Thus, chronic administration of such 'super nitrate' is conceptually very tempting as it is likely that MPC-1011 will improve perfusion of muscle tissue and hence clinical outcome in PAD patients. In order to test this hypothesis, the currently approved drug for PAD, phosphodiesterase III inhibitor cilostazol (Pletal®, 6-[4-(1-cyclo-hexyl-1*H*-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1*H*)- quinolinone) was administered alone or combined with MPC-1011 in an animal model of PAD.

2. Materials and methods

A detailed description of the methods used in this study can be found in Supplementary Materials.

2.1. Animals

Four-week-old male Sprague-Dawley (SD) rats were used for all the experiments. The animals were kept in the Core Rodent Facility at the Department of Physiology, University of Zurich, Switzerland. The food regimen and treatment assignments are explained in detail in Supplementary Materials.

2.2. Surgical procedure of hind-limb ischemia

During surgery, animals were positioned on a heating plate to keep the body temperature constant. Aseptic surgery was performed by a linear incision in the right groin down to the knee. Unilateral hindlimb ischemia will be surgically induced by left femoral artery occlusion as previously described [21]. Detail description of the surgical procedure can be found in the upplementary Materials.

2.3. Surgical procedure and osmotic minipump implantation

Those rats that were randomly assigned to receive either MPC-1011 alone, or in combination with cilostazol, were implanted s.c. with miniosmotic pumps for continuous MPC-1011 delivery (Model 2004, Alzet, Cupertino, CA, USA; length 3 cm, diameter: 0.7 cm, weight: 1.1 g, total displaced volume: 1 ml). Detailed description of the miniosmotic pump implantation can be found in Supplementary Materials.

2.4. Drugs and dosages

After hind limb ischemia ligation, the rats were randomly assigned to receive either MPC-1011 using miniosmotic pump (3.6 mg/day subcutaneously into the back of the animals at the same time when hind limb ligation was induced), cilostazol (100 mg/kg/bi-daily), MPC-1011 plus cilostazol, or placebo. MPC-1011 was kindly provided by mirandaPharmaceuticals, Basel, Switzerland.

2.5. Laser Doppler imaging

For laser Doppler imaging, the rats were anesthetized using isofluorane (5% induction, 1–3% for the maintenance), placed in the prone position on a circulating warm water blanket (37 °C), and positioned under the laser Doppler imager (Moor Instruments, Axminster, UK). Details on laser Doppler imaging procedure and the quantification are described in the Supplementary Materials.

2.6. MicroCT imaging

Directly after the operation and induction of hindlimb ischemia, the animals were transferred into the microCT apparatus (Quantum Fx microCT Imaging System, PerkinElmer, Waltham MA) where the imaging took place. Preparation of the animals for CT imaging and the procedure details can be found in the Supplementary Materials.

2.7. Post mortem analysis

At the end of the experiment (i.e. 28, 14, or 6 days) animals were anesthetized using CO_2 and euthanized by exsanguination and the adductor muscles were isolated and used for immunohistochemistry. Hematoxylin and eosin staining was used for histological examination as described in the Supplementary Materials.

Slides were scanned using digital slide scanner NanoZoomer-XRC12000 (Hamamatsu, Japan) and angiogenic responses were measured by the analysis of capillary density and the ratio of the number of capillaries per muscle fiber. The total number of muscle fibers and capillaries present in 20 fields (40x magnification) per slide was counted and the capillary densities were expressed by numbers of capillaries/mm^2. Arterioles density was quantified in histological sections of the adductor muscle. Results were expressed as number of arterioles/mm^2 of tissue. To check the arteriolar remodeling response to the ischemia, the immunohistochemistry was performed as described above. The measurements of wall and luminal areas and diameter calculation of arterioles were performed in an equal random area for each histological sample. Evaluation of arteriole remodeling was based on the classification described earlier [21].

2.8. Platelet activation assay

After 2 weeks of treatment with MPC-1011, cilostazol, MPC-1011 plus cilostazol or placebo, respectively rat platelets were isolated from 1 ml blood anticoagulated with sodium citrate (9:1) and processed as explained in the Online Supplement, Detailed Methods. Acquisition was done on a FACSCanto (BD Biosciences) and mean fluorescence intensities for 10′000 events recorded and calculated with the FACSDiva software (BD Biosciences).

2.9. VEGF, SDF1α and cGMP levels

Levels of vascular endothelial growth factor (VEGF), SDF1α and cyclic guanosine monophosphate (cGMP) were quantified in homogenates of the tibialis muscle in treated animals 6 days after the operation, and analyzed as described in the Supplementary Materials.

2.10. FACS analysis of proangiogenic endothelial progenitor cells

Mobilization of putative stem cells from bone marrow into the peripheral blood in the placebo and treated groups of the rats was assessed by flow cytometry in whole blood, 6 days after surgery, as described in the Supplementary Materials.

2.11. Endothelial cells scratch assay

Human aortic endothelial cells (HAECs) migration was assessed using the scratch assay in the presence of MPC-1011 and cGMP inhibitor (Ly 83583, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) as explained in the Supplementary Materials.

2.12. Endothelial cells transmigration assay

HAECs, the passage number 7, were cultivated with EGM-2 10% for transmigration assay and were analyzed as described in the Supplementary Materials.

2.13. Statistical analysis

All results are expressed as the mean ± SEM. Comparisons between two groups were performed using Student's t-test or by one-way ANOVA with Bonferroni correction for multiple group comparisons. Blood flow recovery data were analyzed using two-way ANOVA, followed by Bonferroni *post-hoc* analysis. A p-value smaller than 0.05 was considered statistically significant. GraphPad Prism (version 8.4.2, GraphPad Software, San Diego, CA, USA, www.graphpad.com) was used for the statistical analysis.

3. Results

3.1. Blood perfusion in experimental hindlimb ischemia

Laser Doppler imaging was used to assess blood perfusion after induction of hind limb ischemia and randomization of the animals to receive either MPC-1011 alone, cilostazol alone, MPC-1011 plus cilostazol or placebo, respectively (Fig. 1). Successful anatomical removal of the vessels was verified by microCT technology (Supplementary Video). Induction of unilateral hind limb ischemia was associated with a significant reduction of blood flow after 6, 9 and 15 days, as assessed by laser Doppler imaging.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2020.05.012

Of interest, laser Doppler imaging documented increased blood perfusion in rats receiving MPC-1011 (10.5%, 8.4%, and 16.4% *versus* placebo, respectively; $p < 0.05$) or the combination of MPC-1011 plus cilostazol (15.8%, 12.4%, 16.4% *versus* placebo, respectively; $p < 0.05$), while blood flow in those receiving cilostazol only did not differ from placebo-treated animals (Fig. 2A).

Similarly, the area under the curve (AUC) was increased in MPC-1011- or combination-treated rats (by 44.2% and 49.7%, respectively; $p < 0.05$; MPC-1011 *vs.* placebo and MPC- 1011 versus cilostazol, respectively; by 54.2% and 60.1%; $p < 0.05$, combination *vs.* placebo and combination *vs.* cilostazol, respectively; Fig. 2B).

3.2. Pro-angiogenic effects of MPC-1011

After 2 weeks of treatment, capillary number was increased in rats receiving MPC-1011 and in combination-treated groups as compared to placebo or cilostazol (20.4% and 33.8%, $p < 0.05$ MPC-1011 *vs.*

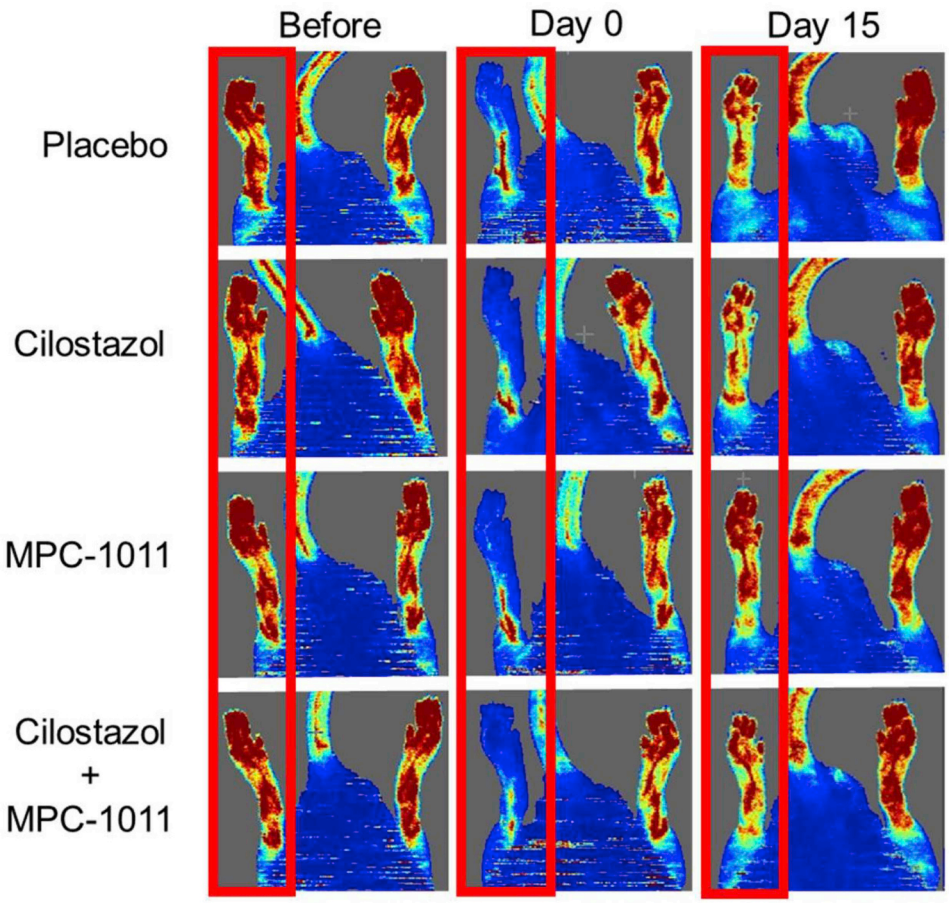

Fig. 1. Representative image of blood perfusion upon hindlimb ischemia.
Representative image of blood perfusion in rats treated with placebo, cilostazol, MPC-1011, and cilostazol plus MPC-1011 before the surgery, immediately after the surgery (day 0) and 15 days after the surgery in a color-coded representation where the right hindlimb was ligated (blue color) and left hindlimb was not operated (red color) and latter serves as an internal standard for blood perfusion determination.

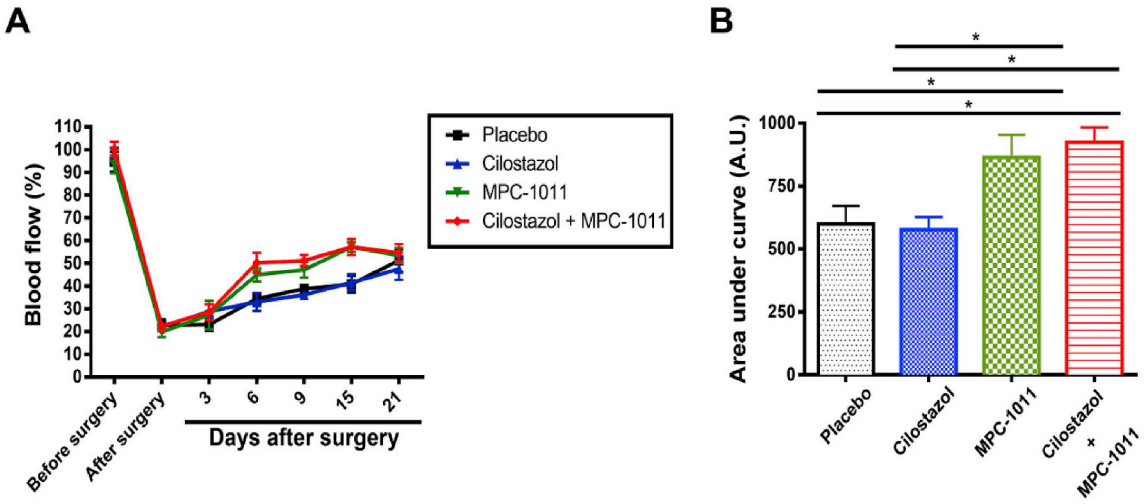

Fig. 2. Blood perfusion in four treatment arms of rats that underwent hindlimb ischemia.
Blood perfusion in ligated hindlimb expressed as % blood flow compared to contralateral limb (A) and area under the curve in four treatment arms (B). Blood flow was measured immediately before and after surgery using laser Doppler imaging and was measured every third day for a total duration of 3 weeks. Data are presented as mean ± SEM. *$p < 0.05$. Bonferroni-adjusted p values are shown. N = 9–11, where N represents an individual animal.

placebo and MPC-1011 *vs.* cilostazol, respectively; 32.6% and 47.4%, $p < 0.01$, combination treatment *vs.* placebo and combination treatment *vs.* cilostazol, respectively) (Fig. 3A and Supplementary Fig. 1A).

Similarly, after 4 weeks, the number of capillaries was increased in MPC-1011- and combination-treated groups as compared to those receiving placebo or cilostazol alone (24.6% and 28.7%, $p < 0.01$; MPC-1011 *versus* placebo and MPC-1011 *versus* cilostazol, respectively; 25.5% and 29.6%, $p < 0.01$; combination *versus* placebo and combination *versus* cilostazol, respectively; Fig. 3B).

The effect of MPC-1011 and combination therapy was highly specific and only affected the operated hind limb, while no changes in capillary number were detected in the contralateral (non-operated) limbs (Supplementary Fig. 1B-D).

3.3. Pro-arteriogenic effects of MPC-1011

After 4 weeks, the number of arterioles was increased in MPC-1011- and combination-treated groups as compared to placebo or cilostazol alone (54.5% and 42.3%, respectively, $p < 0.05$, MPC-1011 *vs.* placebo and MPC-1011 *vs.* cilostazol, respectively; 46.1% and 34.6%, $p < 0.05$, combination *vs.* placebo and combination *vs.* cilostazol, respectively), while no change was observed in contralateral, non-operated limb (Fig. 3C; Supplementary Fig. 2A and B).

3.4. MPC-1011 and vascular remodeling

After 6 days of treatment, diameter of the arterioles was increased in MPC-1011- and combination-treated groups as compared to placebo or cilostazol alone (21.1% and 17.6%, $p < 0.05$, MPC-1011 *vs.* placebo and MPC-1011 *vs.* cilostazol, respectively; 28.2% and 24.5%, $p < 0.01$ and $p < 0.05$, combination *vs.* placebo and combination *vs.* cilostazol, respectively; Supplementary Fig. 3A). At 6 days, the wall area size was not affected by any of the treatments (Supplementary Fig. 3B), and the ratio of arterial wall area and luminal area was reduced in MPC-1011 and combination-treated as compared to cilostazol (22.8%, $p < 0.05$, MPC-1011 *vs.* cilostazol and 19.3%, $p < 0.05$, combination *vs.*

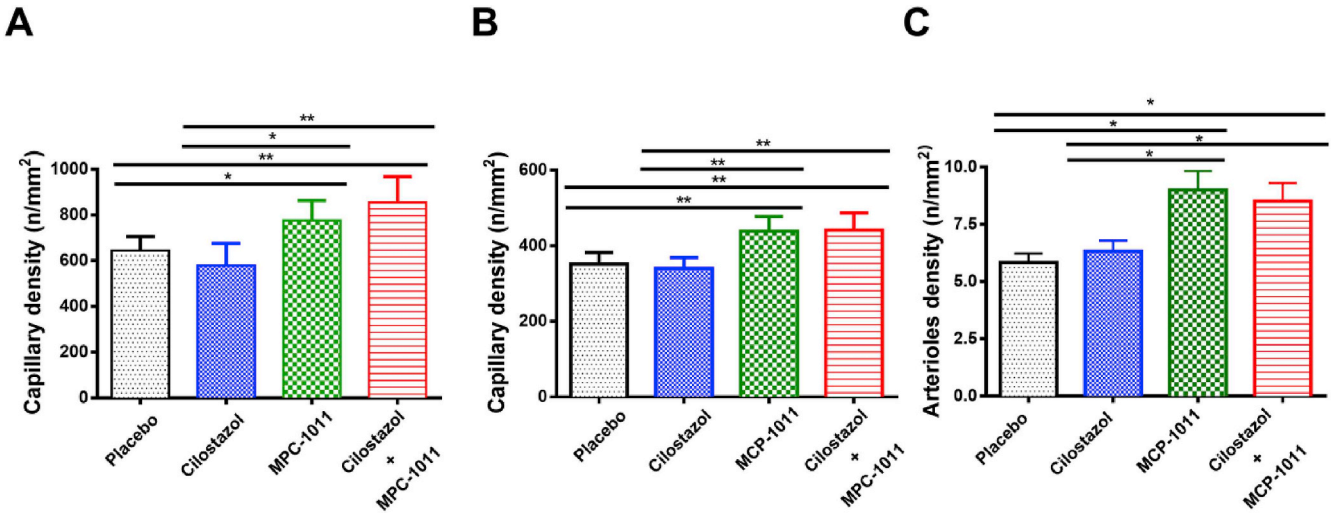

Fig. 3. Capillary and arteriole density in four different treatment groups upon hindlimb ischemia.
Capillary density, expressed as number of capillaries per mm^2, in ligated limb after 2 (A) and 4 (B) weeks of treatment; (C) arterioles density in ischemic limbs from four experimental groups. Arterioles density was expressed as number of arterioles per mm^2 in ligated limb after 4 weeks of treatment. The total number of muscle fibers and capillaries present in 20 fields (40x magnification) per slide was counted and the capillary densities were expressed by numbers of capillaries/mm^2-. Data are presented as mean ± SEM. *$p < 0.05$ and **$p < 0.01$. Bonferroni-adjusted p values are shown. N = 4–5, where N represents an individual animal.

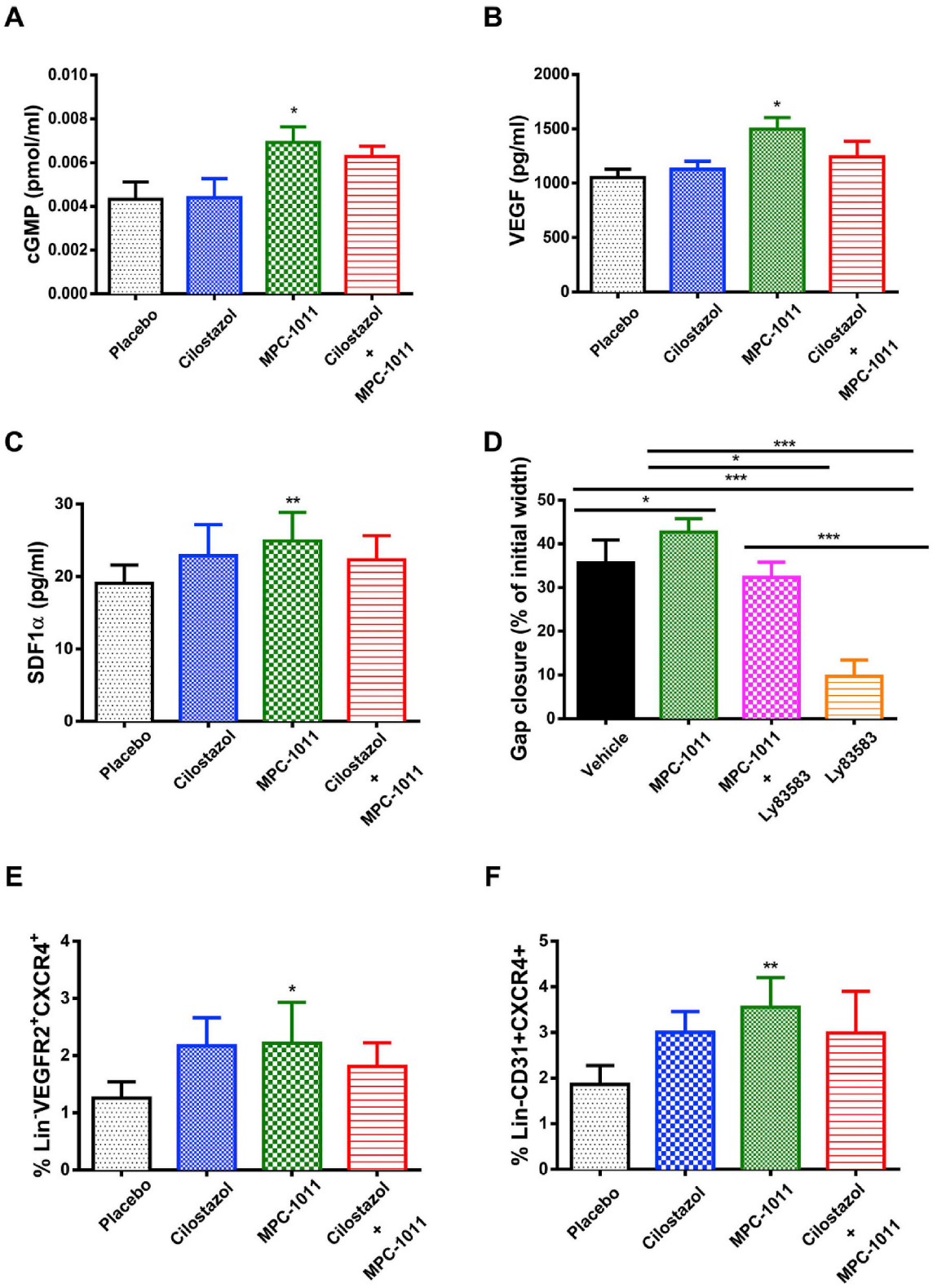

Fig. 4. Proangiogenic response *in vivo* and in *vitro*.
cGMP- (A), VEGF- (B) and SDF1α-levels (C) in homogenates of tibialis muscle from rats treated for 6 days with either placebo, MPC-1011 alone, cilostazol alone, or MPC- 1011 plus cilostazol; (D) *in vitro* effects of MPC-1011 in the presence or absence of cGC inhibitor, LY83583, on gap closure; population of $Lin^-/VEGFR2^+/CXCR4^+$ (E) and $Lin^-/CD31^+/CXCR4^+$ (F) cells from whole rat blood in four treatment arms. Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Bonferroni-adjusted p values are shown. N = 7–9 where N represents an individual animal. For cell culture experiments, N = 3–4 where N represents a biological replicate.

cilostazol, respectively; Supplementary Fig. 3C).

Similar effects were observed at 2 weeks: diameter of the arterioles was increased in MPC-1011- and combination-treated groups as compared to placebo or cilostazol alone (21.0% and 14.2%, $p < 0.05$, MPC-1011 *vs.* placebo and MPC-1011 *vs.* cilostazol, respectively; 26.6% and 19.6%, $p < 0.01$ and $p < 0.05$, combination *vs.* placebo and combination *vs.* cilostazol, respectively) (Supplementary Fig. 4A), while the wall area remained unaffected in all groups (Supplementary Fig. 4B). Accordingly, the ratio of arterial wall area and luminal area was reduced with MPC-1011 and combination-treated also at that time point as compared to placebo or cilostazol alone (23.6% and 38.0%, $p < 0.05$ and $p < 0.001$, MPC- 1011 *vs.* placebo and MPC-1011 *vs.* cilostazol, respectively; 19.2% and 34.6%, $p < 0.05$ and $p < 0.01$, combination *vs.* placebo and combination *vs.* cilostazol, respectively); Supplementary Fig. 4C).

After 4 weeks, measured diameter of the arterioles was increased with MPC-1011 and combination-treated as compared to placebo or cilostazol alone (23.1% and 27.0%, $p < 0.05$, MPC-1011 *vs.* placebo and MPC-1011 *vs.* cilostazol, respectively; 25.0% and 28.8%, $p < 0.05$, combination *vs.* placebo and combination *vs.* cilostazol, respectively; Supplementary Fig. 5A). The wall area size in all groups remained unaffected (Supplementary Fig. 5B), but the ratio of arterial wall area and luminal area was reduced with MPC-1011 and combination-treated as compared to placebo or cilostazol alone (23.9% and 34.1%, $p < 0.01$ and $p < 0.001$, MPC-1011 *vs.* placebo and MPC-1011 *vs.* cilostazol, respectively; 22.0% and 32.3%, $p < 0.01$ and $p < 0.001$, combination *vs.* placebo and combination *vs.* cilostazol, respectively; Supplementary Fig. 5C).

The effects of remodeling by MPC-1011- and combination-treatment were highly specific as no changes in the wall/luminal area or arteriolar diameter were noted in the contralateral non-ischemic limbs (Supplementary Fig. 6).

3.5. MPC-1011 increases cGMP, VEGF and SDF1α levels and stimulates $CXCR4^+/VEGFR2^+$ proangiogenic EPCs

After 6 days, MPC-1011 increased tissue cGMP levels in homogenates of tibialis muscle by 60.2% compared to placebo (Fig. 4A). Muscle VEGF levels were increased by MPC-1011 by 42.2% (Fig. 4B), while SDF1α levels were increased by 30.7% as compared to placebo (Fig. 4C).

When HAECs were incubated with the sGC inhibitor LY83583, the capacity gap closure decreased by 72.9% as compared to vehicle ($p < 0.001$), while MPC-1011 alone increased gap closure by 19.6% as compared to vehicle-treated cells. In cells pre-incubated with LY83583, MPC-1011 led to a 3.4-fold increase in gap closure as compared to the inhibitor alone ($p < 0.001$; Fig. 4D).

FACS analysis indicated in serum of MPC-1011-treated rats a 76.8% increase of $Lin^-/VEGFR2^+/CXCR4^+$ cell population *vs.* placebo (Fig. 4E), while $Lin^-/CD31^+/CXCR4^+$ cells population was increased for a 92.0% *vs.* placebo (Fig. 4F).

3.6. Endothelial cell transmigration assay

Migration of endothelial cells in modified Boyden chambers against full medium as a chemoattractant was increased by MPC-1011 by 19.0% ($p < 0.01$, *vs.* vehicle), while transmigration was reduced by --31.7% in the presence of the soluble guanylate cyclase inhibitor, LY83583 (10^{-5} M; $p < 0.001$ *vs.* MPC-1011-stimulated migration) (Fig. 5A). If MPC-1011 was used as a chemoattractant, migration of endothelial cells increased by 92.0% ($p < 0.001$, *vs.* vehicle), while LY83583 reduced transmigration against MPC-1011 by 18.0% ($p < 0.01$ *vs.* MPC-1011 alone; Fig. 5B).

3.7. MPC-1011 prevents platelet aggregation

Flow cytometry of the surface marker P-selectin in platelets in a resting, unstimulated state, indicated that in MPC-1011-treated rats its expression level was reduced by 44.0% as compared to placebo-treated rats (Supplementary Fig. 7; $p < 0.05$).

4. Discussion

To the best of our knowledge, we here for the first time show important effects of the NO donor MPC-1011 on blood flow, angiogenesis, arteriogenesis, tissue remodeling in a rat model of PAD *in vivo* supported by *in vitro* studies on cell proliferation and transmigration. Indeed, after femoral artery ligation MPC-1011 increased blood flow,

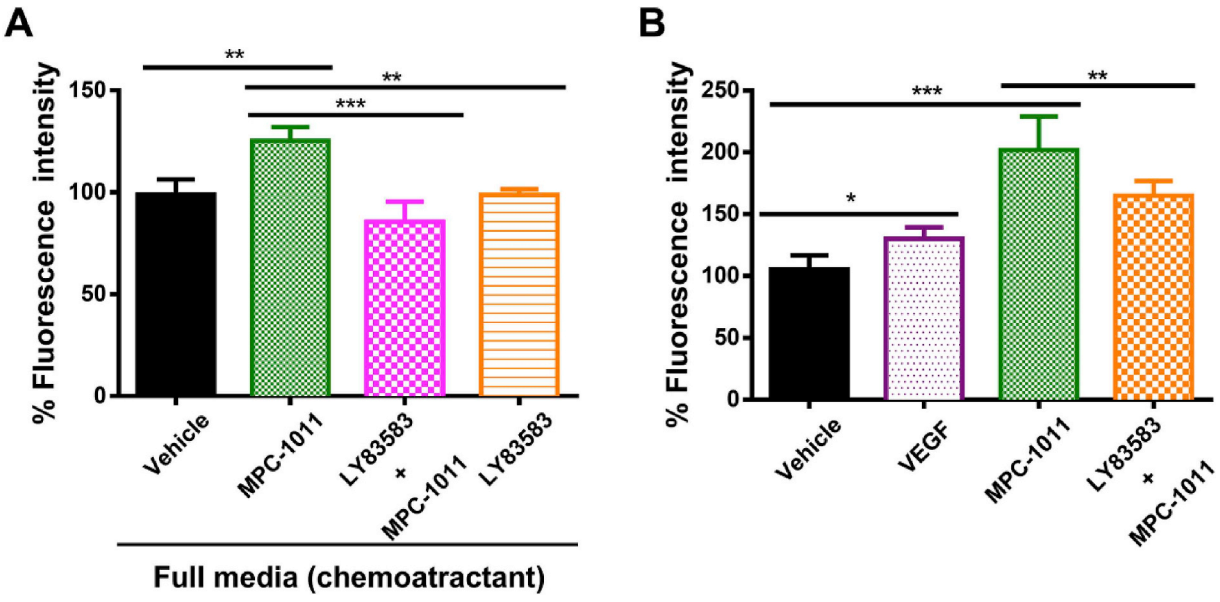

Fig. 5. Transmigration assay of HAECs.
Transmigration of HAECs pretreated with MPC-1011 alone, MPC-1011 plus LY83583, and LY83583 alone against full medium as a chemoattractant (A), and transmigration of untreated HAECs when VEGF alone, MPC-1011 alone or MPC-1011 plus LY83583 was used as chemoattractants (B). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. Bonferroni-adjusted p values are shown. N = 4–8 where N represents a biological replicate.

the number of capillaries as well as arterioles, induced positive vascular remodeling, and increased tissue levels of VEGF, cGMP, and SDF1α. At the cellular level MPC-1011 facilitated transmigration of endothelial cells, a crucial component of the angiogenic process. Finally, MPC-1011 reduced the expression of P-selectin in platelets as well as platelet aggregation.

NO is a central constituent of vascular homeostasis under physiological conditions and particularly during tissue ischemia [22]. Hence, NO substitution has become a promising pharmacological treatment for cardiovascular conditions [23,24]. Although many structurally and chemically diverse NO-donor compounds have been synthesized and used widely in experimental studies, no NO-donor has been approved for use in the clinic, mostly due to development of nitrate tolerance leading to loss of potency and induction of vascular oxidative stress.

MPC-1011 (2-nitrooxyethylammoniumnitrate, 2-aminoethyl nitrate [AEN], itramin tosylate) belongs to the class of organic nitrates that mediate relaxation by generation of NO or related species. It has been marketed for the indication of angina pectoris between 1957 and 1969 under the trade name Nilatil ® (Pharmacia) [25] and was shown to have favourable properties *in vivo* and *in vitro* [19,20], suggesting clinical superiority over established compounds such as nitroglycerine/glyceryl trinitrate (GTN) [26–29]. MPC-1011 has a potent vasodilatory properties *ex vivo*, it does not lead to oxidative stress in isolated heart mitochondria [30], and unlikely to GTN, biotransformation of MPC-1011 is not mediated by mitochondrial aldehyde dehydrogenase (ALDH-2) [19].

To test the hypothesis that use of the NO donor MPC-1011 would be better than currently existing PAD treatment options such as cilostazol, we used a rat model of an induced hindlimb ischemia. Since PAD has traditionally been identified as a male-dominant disease, we used male rats for inducing hindlimb ischemia. It is worth mentioning that some population trends and studies [31,32] suggest that women are affected at least as often as men, and thus, gender-based differences might not play crucial role in presentation of PAD. We performed computer tomography imaging using ExiTron™ nano 12000 nanoparticles and successfully demonstrated removal of the vessels *in vivo*. Further, using laser Doppler imaging we demonstrated a marked and persistent increase in blood perfusion over - 2 weeks testing period of MPC-1011 administration either alone or in combination with cilostazol, while no increase in blood perfusion was achieved by cilostazol alone. The effect of MPC-1011 in our rodent hindlimb ischemia model was a specific angiogenic response to reduced limb perfusion, since the effects were seen only in ischemic tissue and not in the contralateral, not operated leg. Our data are in line with the improved revascularization in chronic ischemia upon dietary nitrate supplementation [33] as well as with the enhancement of ischemia-induced angiogenesis and arteriogenesis upon chronic nitrite administration [34].

Significantly reduced NO bioavailability is the main factor to the impaired endothelial function that leads to abnormal vascular reactivity. Angiogenic potential of endothelial cells depends on biosynthesis and release of well-established angiogenic molecules, including vascular cell adhesion molecule (VCAM), prostacyclin and VEGF, among others. Circulating VCAM-1, a biomarker of endothelial dysfunction, in patients with PAD is elevated due to its function in mediating adhesion of immune cells to the vascular endothelium in the process of endothelial dysfunction and inflammation [35]. Similarly, prostacyclin biosynthesis and release by endothelial progenitor cells (EPCs) is shown to be a crucial angiogenic phenomenon that contributes to their regenerative function [36]. Yet another important angiogenetic growth factor that affects proliferation, migration, survival and permeability of endothelial cells is VEGF [37]. It upregulates the expression of endothelial NO synthase (eNOS) and stimulates the release of NO that plays a critical role in its angiogenic action [38]. Thus, NO is the crucial downstream mediator of VEGF-induced endothelial cell proliferation and migration [39]. In turn, NO activates soluble guanylate cyclase (sGC), resulting in elevated cGMP levels that can augment ischemia-induced angiogenesis. cGMP is a secondary messenger which leads to vascular smooth muscle cells relaxation, vasodilation and congruently increased blood flow. In line with this concept, cGMP tissue levels were enhanced in MPC-1011 treated animals demonstrating that the NO-donor – as other organic nitrates - increases cGMP levels at sites of ischemia [40]. Of note, in endothelial cells the soluble guanylyl cyclase inhibitor LY83583, abolished VEGF-induced angiogenesis as demonstrated by the gap-closure assay. The propensity of the endothelial cells to migrate and seal the gap was markedly reduced by LY83583 in accordance with its known inhibitory effects on VEGF-mediated angiogenic responses [41]. The migration and proliferation of the endothelial cells upon exposure to MPC-1011 was also examined in the Boyden chamber. Our data show unequivocally that MPC-1011 improved cellular migration and proliferation *in vitro*. Further, these processes must be at least partially mediated by cGMP, since LY83583 again reduced the capacity of cells pre-incubated with MPC-1011 to proliferate and migrate against the chemoattractant gradient. These results are in line with already published observation on stimulatory effects of nitrites on endothelial proliferation and migration in an NO/VEGF- dependent manner *in vitro* [42] and *in vivo* [34].

VEGF is also involved in the recruitment of bone marrow-derived cells to ischemic tissue by stimulating release of stromal-derived factor (SDF)-1α from platelets. SDF-1α promotes retention of bone marrow-derived cells at damaged sites via its receptor, CXC chemokine receptor type 4 (CXCR4) [43]. The mobilization of cells that express receptors for VEGF and SDF-1α [44] to ischemic tissues is critical for angiogenesis. This was supported by our FACS analysis of whole blood sorted for different cell surface receptors where we showed an increase of the $CXCR4^+$ and $VEGFR2^+$ cell populations upon MPC-1011 treatment. Thus, MPC-1011 not only stimulates VEGF release, but simultaneously affects the release of SDF-1α which in turn acts on its cognate receptor CXCR4 and mobilizes cells into the area of injury and promotes revascularization of damaged tissues. A potential limitation of the effects described here is the fact that certain solid tumors are able to grow and metastatically spread due to an abnormal VEGF expression [45], an effect that would be addressed during clinical development of MPC-1011.

Finally, we observed a reduction in platelet activation in rats treated with MPC-1011 as assessed by platelet P-selection expression in a non-stimulated state. This corroborates evidence that NO donors inhibit platelet aggregation *in vitro* and *in vivo* via the NO-cGMP pathway [46] an effect that would also be therapeutically favourable in the PAD population prone to atherothrombotic complications in the leg as well as the heart and brain.

In conclusion, we demonstrate that chronic treatment with the nitric oxide donor MPC- 1011 has a high potential for selectively restoring perfusion to chronically ischemic peripheral tissues as it stimulates various aspects of vascular remodeling such as angiogenesis and arteriogenesis in a NO-dependent manner without tolerance.

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CRediT authorship contribution statement

Brigida Gomes de Almeida Schirmer: Investigation, Formal analysis, Validation. **Margot Crucet:** Investigation. **Simona Stivala:** Investigation. **Goran Vucicevic:** Investigation. **Luciola da Silva Barcelos:** Supervision. **Paul M. Vanhoutte:** Supervision, Writing - review & editing. **Giovanni Pellegrini:** Investigation. **Giovanni G. Camici:** Resources. **Petra Seebeck:** Resources. **Svende Pfundstein:** Investigation. **Sokrates Stein:** Supervision. **Francesco Paneni:** Investigation. **Thomas F. Lüscher:** Conceptualization, Resources, Supervision, Writing - review & editing, Project administration, Funding acquisition. **Branko Simic:** Conceptualization, Methodology, Validation, Resources, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

TFL is a member of the advisory board at mirandaPharmaceuticals. The other authors have nothing to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2020.05.012.

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